*GENETIC FUNCTIONS OF THE CHLOROPLAST OF CHLAMYDOMONAS REINHARDI: EFFECT OF RIFAMPIN ON CHLOROPLAST DNA-DEPENDENT RNA POLYMERASE**

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Abstract.—The effect of rifampin, an inhibitor of bacterial DNA-dependent RNA polymerase, was studied in *Chlamydomonas reinhardi*. It was shown, *in vivo* and *in vitro*, that chloroplast-located, but not nuclear, DNA-dependent RNA polymerase is inhibited by this drug. The inhibition of chloroplast RNA polymerase results in the inhibition of chloroplast rRNA synthesis, and thus in the loss of chloroplast ribosomes. The ability to carry out photosynthesis is also lost after prolonged heterotrophic growth in the presence of rifampin, but cell division and chloroplast replication are not affected. It is proposed that chloroplast DNA contains information for chloroplast rRNA, but this DNA does not have the information for chloroplast DNA polymerase. Moreover, the DNA polymerase is not synthesized on chloroplast ribosomes.

The nature and amount of information encoded in chloroplast DNA is not known. A possible approach to this problem is to study *in vivo* the effect of specific inhibitors of chloroplast DNA-dependent RNA polymerase.

It was recently shown that antibiotics of the rifamycin group specifically inhibit DNA-dependent RNA polymerase (E.C. 2776) of bacterial, but not mammalian, origin.[1–4] The inhibitors act by specifically binding to the enzyme itself, and not to the DNA template.[5] The effect of this inhibitor on the DNA-dependent RNA polymerase of chloroplasts of *Chlamydomonas reinhardi* is reported in this paper.

Materials and Methods.—UTP, GTP, CTP, and ATP were purchased from Calbiochem, α^{32}P-ATP from ICN (1 c/mmole), adenine-8-^{14}C from Schwarz (54 mc/mmole), pyruvate kinase from Sigma, ribonuclease (crystallized) and calf thymus DNA from Worthington, and actinomycin D from Merck. Rifampin, 3-(4-methylpiperazinyliminomethyl) rifamycin SV, was a gift from Dow Chemical.

Wild-type *C. reinhardi* (strain 137c, mating type plus) was used in all experiments. The cells for chloroplast isolation and for nucleic acid labeling experiments were grown at 25° on minimal medium[6] for 50 hr in a 3-liter volume. The cultures were agitated by bubbling with 5% CO_2 in air. The light intensity was 4000 lux. Cells grown in the presence of rifampin were cultured on an acetate-containing medium.[7] Because of the instability of the drug to light, the cells were grown in the dark or under reduced light intensity when experiments were carried out longer than 24 hr.

A new method of chloroplast isolation which yields highly purified and intact chloroplasts was developed. Six liters of cells were collected and washed three times with cold buffer I (0.25 *M* sucrose, 0.05 *M* Tris-HCl buffer, pH 8.0, 1×10^{-3} *M* Mg EDTA, and 2×10^{-2} *M* $MgSO_4$) and then twice with cold buffer II (0.5 *M* sucrose, 0.05 *M* Tris-HCl buffer, pH 8.0, 2×10^{-2} *M* $MgSO_4$, 0.25% BSA, 2×10^{-3} *M* 2-mercaptoethanol). They were then suspended in cold buffer II at the ratio of 1 vol of buffer to 2 vol of pelleted cells and were broken in a French pressure cell at a pressure of 35 kg/cm^2. The suspension of broken cells was diluted with 3 vol of cold buffer II and centrifuged in 20-ml vol for 1.5 min at 500 × *g* in a Sorvall RC-2B centrifuge with an SS-34 rotor. The super-

natant was collected and centrifuged for 7 min at 480 × g. These centrifugations were repeated three times for 2 min each, until the resuspended pellet contained only a mixture of protoplasts and chloroplasts.

Separation of chloroplasts from protoplasts was achieved by centrifugation on a discontinuous sucrose gradient in buffer II. The gradient contained four 5-ml layers: 1.5, 1.75, 2.0, and 2.5 M sucrose. Five ml of the chloroplast-protoplast mixture were layered on top of each of the gradients, which were then centrifuged for 60 min at 24,000 rpm in an SW-25-1 rotor in a Beckman model L centrifuge. Three distinct green bands are formed. The first band, located on the border of the 1.5 and 1.75 M sucrose layers, contains chloroplasts with damaged outer membranes. The second band is located between the 1.75 and 2.0 M sucrose layers and contains intact chloroplasts. The third band is formed on the top of the 2.5 M sucrose layer and contains protoplasts. Each of the three bands was collected with an ISCO gradient fractionator and resuspended in 6 vol of buffer II. These suspensions were centrifuged three times, for 2 min each, at 480 × g (as described above) in order to remove residual small fragments.

The purity of the final chloroplast fraction was determined by examination with both the light and electron microscope and by treatment with 0.01% Triton X-100, which causes the immediate lysis of chloroplasts but does not affect protoplasts and cell fragments. The chloroplast fraction is pure by both criteria.

Reaction mixtures for the determination of DNA-dependent RNA polymerase activity in isolated chloroplasts and in protoplasts contained in 1 ml the following (in μmoles): Tris-HCl buffer, pH 8.0, 50; $MgSO_4$, 20; cysteine, pH 7.0, 3; and each of ribonucleoside triphosphates (UTP, GTP, and CTP), 1. Also present were 5 μc α^{32}P-ATP (specific activity, 625 μc/μmole), an ATP-generating system containing 40 μmoles phosphoenolpyruvate and 0.03 mg pyruvate kinase, and from 1×10^6 to 1×10^7 chloroplasts or protoplasts. The reaction mixtures were adjusted to a final volume of 1 ml with 0.5 M sucrose in 0.05 M Tris-HCl buffer, pH 8.0. The reactions were started with the addition of chloroplasts or protoplasts and were run at 28°. Samples (0.1 ml) were withdrawn from each reaction mixture at 1-min intervals and placed in tubes containing 0.07 ml of a mixture of detergents (15% sodium lauryl sulfate, 7.5% Triton X-100 dissolved in SSC). This solution terminates the reaction and causes the immediate lysis of chloroplasts. To achieve lysis of protoplasts, it was necessary to heat the detergent mixture to 70°. The samples were applied to 2.3-cm Whatman no. 3 discs, washed according to a procedure described by Bollum,[8] and counted with the aid of a Nuclear-Chicago gas-flow counter (40% efficiency); 99% of the counts resulting from the described assay were base-hydrolyzable (0.3 N NaOH, 37°, 16 hr).

The ability of whole cells to incorporate adenine-8-^{14}C into the acid-precipitable fraction was taken as an indication of their *in vivo* RNA polymerase activity. Less than 0.1% radioactivity was incorporated into DNA during the time the reaction was run (1.5 hr). The reactions were run at 25° at a light intensity of 4000 lux. Cells from nonsynchronized cultures were harvested during their exponential phase of growth and suspended in minimal medium containing 0.01 μc/ml adenine-8-^{14}C (specific activity 54 mc/mM) at a concentration of 1×10^6 cells/ml. Samples of 0.1 ml were withdrawn at various times throughout the experiment and placed in 0.05 ml of detergent mixture (15% sodium lauryl sulfate, 4% lauryl trimethylammonium bromide, 8% Triton X-100, and 4% triisopropylnaphthalenesulfonic acid sodium salt). The samples were kept at 95° in a water bath for 10 min. Under these conditions the cells were immediately lysed. The samples were prepared for counting by the method described above, except that a liquid scintillation counter was used.

Nucleic acids were isolated by a modified Kirby[9] method. The product of the reactions was chromatographed on a MAK column by using a modified Mandell and Hershey[10] column as described in a forthcoming publication (Hastings and Surzycki). Ribosomal RNA's were fractionated by polyacrylamide gel electrophoresis as described by Loening.[11] DNA isolation and CsCl density centrifugation were carried out as described by Chiang and Sueoka.[12]

Photosynthetic CO_2 fixation was measured by a titrimetric method.[13]

Results.—The effect of rifampin on RNA synthesis *in vivo* was tested by measuring the incorporation of adenine-8-^{14}C into a cold trichloroacetic acid-insoluble product (see *Materials and Methods*). Two identical reaction mixtures were incubated for 30 minutes, at which time rifampin, dissolved in 0.05 *M* phosphate buffer, pH 6.0, was added to one of the reaction mixtures. An equal volume of the same buffer was added to the other reaction mixture. RNA synthesis was followed for an additional 80 minutes.

Rifampin, at a concentration of 250 μg/ml, was found to inhibit RNA synthesis in whole cells by only 44 per cent. Increasing the concentration of the antibiotic did not increase the percentage of inhibition.

RNA polymerase activity in chloroplasts was assayed as described in *Materials and Methods*. The initial rate of incorporation and the final yield of incorporation at the time the reaction reaches completion are a linear function of the number of chloroplasts present in the reaction mixture. Unlike the situation in mitochondria,[14] the incorporation is inhibited by the addition of ribonuclease (10 μg/ml). Omitting magnesium ions, or a ten-minute preincubation in the presence of actinomycin D, inhibits the reaction. The addition of actinomycin D during the course of the reaction also stops the reaction immediately.

The rates of incorporation by broken chloroplasts are less than the rates obtained from the intact chloroplasts. However, the total incorporation observed when the reaction reaches a plateau is similar in all the experiments (Table 1). The addition of native or denatured calf-thymus DNA does not affect the rate or the total incorporation of ATP by chloroplasts taken from either of the two bands. At a minimum this incorporation is equivalent to 1.6×10^6 RNA bases per chloroplast calculated with the assumption that the RNA product formed contains 32.7 per cent adenine. This is the percentage of adenine in *C. reinhardi* chloroplast DNA[15] which contains about 4.6×10^6 bases.[12]

The RNA polymerase reaction did not occur when all the nucleoside triphosphates were omitted from the reaction mixture (Fig. 1). The omission of UTP altered both the initial rate and the final level of ATP incorporation. The omission of CTP or GTP, in contrast, did not affect the rate of RNA synthesis, but caused the reaction to stop prematurely.

The above observations can be interpreted as follows: (1) The chloroplast contains a considerable pool of UTP. This pool allows RNA synthesis to proceed in the absence of added UTP if the other three nucleoside triphosphates are supplied, but the relatively low concentration of UTP in the endogenous pool limits the rate of the reaction. (2) The chloroplast does not have an appreciable pool of either GTP or CTP since RNA synthesis is virtually abolished if these nucleoside triphosphates are omitted from the reaction mixture, even in the presence of ATP and given a considerable pool of UTP. Omitting only GTP or

TABLE 1. *$\alpha^{32}P$-ATP incorporation by isolated chloroplasts.*

Expt. no.	Layer from sucrose gradient	Initial rate ($\mu\mu$moles/10^6 chloroplasts/min)	Total incorporation ($\mu\mu$moles/10^6 chloroplasts)
1	1	0.0385	0.540
2	1	0.0422	0.575
1	2	0.0760	0.538
3	2	0.0750	0.632

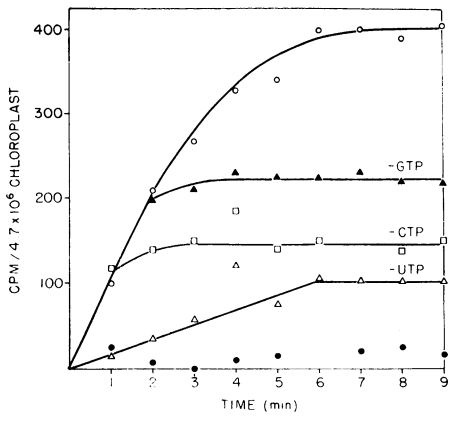

FIG. 1.—Kinetics of RNA synthesis in isolated chloroplasts. O, Total reaction mixture as described in *Methods;* ▲, reaction mixture minus GTP; □, reaction mixture minus CTP; △, reaction mixture minus UTP; ●, reaction mixture minus GTP, CTP, and UTP.

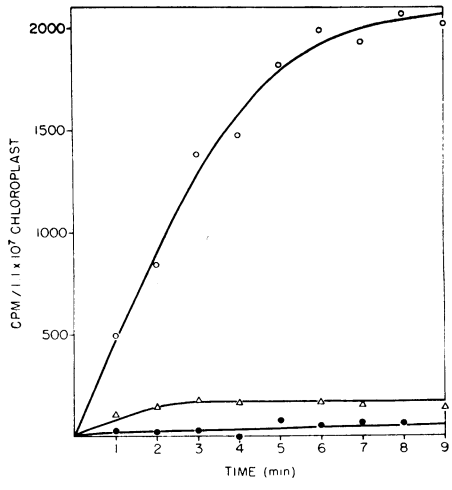

FIG. 2.—Effect of rifampin on RNA synthesis in isolated chloroplasts. O, Total reaction mixture as described in *Methods;* △, chloroplasts preincubated with 50 μg/ml of rifampin; ●, chloroplasts preincubated with 100 μg/ml of rifampin.

CTP does not affect the rate of the reaction, but causes the reactions to stop earlier. CTP and GTP do not appear to be required for the incorporation of about 20 and 28 per cent, respectively, of the α^{32}P-ATP (Fig. 1); transcription of chloroplast DNA therefore may begin and proceed first through an A-T rich part of the DNA. (3) The unequal requirement for GTP and CTP, which is always seen in the same proportions, seems to indicate that the transcribed DNA molecules are relatively simple and almost identical and that transcription proceeds along only one of their complementary strands.

The ^{32}P-labeled product of the DNA-dependent RNA polymerase reaction was isolated and chromatographed on a MAK column together with unlabeled carrier RNA from *C. reinhardi.* All (99%) of the radioactivity was eluted together with the high-molecular-weight RNA at salt concentrations of 1.2 to 1.4 *M.*

Assay of RNA polymerase in protoplasts revealed that, in contrast to the chloroplasts, they were able to incorporate α^{32}P-ATP in a nearly linear fashion for a much longer time: The rate of the reaction after 20 minutes was almost the same as after ten minutes. No RNA synthesis occurred if the protoplasts were preincubated with actinomycin D for ten minutes before starting the reaction, or if all three nucleoside triphosphates were omitted from the reaction mixture. If either UTP or GTP was left out of the reaction mixture, RNA synthesis proceeded at a limited rate, indicating that pools of these two nucleoside triphosphates were present, whereas omitting CTP inhibited the reaction completely.

The addition of rifampin (100 μg/ml) to isolated chloroplasts prior to starting the reaction (at 0°C) inhibits RNA synthesis completely (Fig. 2). Concentra-

tions of less than 50 μg/ml have very little effect. The addition of rifampin after the reaction is started (2 min), at concentrations of 100 to 500 μg/ml, does not visibly inhibit the reaction. This result is in agreement with the findings of Mizuno *et al.*[4] that a DNA-enzyme complex after initiation is insensitive to the inhibitor. It is therefore concluded that the mechanism of the action of rifampin on chloroplast DNA-dependent RNA polymerase is very similar to its action on bacterial RNA polymerase in that it can bind to the polymerase complex and prevent initiation but not polymerization processes of RNA synthesis. Partially broken and extensively washed chloroplasts would be expected to retain only the DNA-bound enzyme actively engaged in polymerization. Accordingly, rifampin should not have any affect on these chloroplasts; this was shown to be the case.

Preincubation of protoplasts at 0° with rifampin before starting the reaction also partially inhibits the incorporation of ATP. However, the maximum inhibition achieved is never more than 44 per cent of the total activity and, as was found in the case of whole cells, increasing the concentration of rifampin as high as 500 μg/ml does not produce any further inhibition. Table 2 and Figure 3

TABLE 2. *Rate of α^{32}P-ATP incorporation by isolated protoplasts.*

	$\mu\mu$moles/10^6 protoplast/min	Percentage of A
(*A*) Protoplasts (chloroplast + nucleus)	0.1430	100
(*B*) Rifampin-treated protoplasts (presumably nucleus minus chloroplast)	0.0805	56.2
(*C*) Chloroplasts (calculated as *A* minus *B*)	0.0625	43.8

present the results from one of these experiments. The calculated initial rate of the rifampin-sensitive reaction is equal to 0.625 $\mu\mu$mole ATP/min/10^6 chloroplasts which is similar to the rates obtained in the reactions with purified chloroplasts (see Table 1). The rifampin-insensitive RNA synthesis in the protoplasts proceeds in a nearly linear fashion for a much longer time (over 20 min) than the reaction observed in isolated chloroplasts. It is therefore highly probable that the rifampin-sensitive synthesis of RNA in protoplasts is identical to the RNA-synthesizing system found in purified chloroplasts. This system is responsible for 44 per cent of the RNA synthesis (as measured by ATP incorporation) found in isolated protoplasts and for the same amount of RNA synthesis in intact cells.

To test the effect of rifampin *in vivo*, the cells were grown heterotrophically for five generations in the presence of 250 μg/ml rifampin. A control culture was grown in the same way, but without the addition of rifampin. The nucleic acids from these cells were isolated and fractionated by polyacrylamide gel electrophoresis (Fig. 4). The two RNA peaks, designated 25*S* and 18*S*, represent ribosomal RNA from cytoplasmic ribosomes, and the two peaks designated 23*S* and 16*S* RNA are from the chloroplast ribosomes (Fig. 4, *top*). Their electrophoretic mobilities are related to sedimentation constants with ribosomal RNA from *E. coli* as a standard. The cells in the control culture have all four peaks. The cells which were grown in the presence of rifampin lack the 23*S* and 16*S* peaks, indicating that their chloroplast rRNA synthesis was inhibited. Moreover, it has been possible to show that when exponentially growing cells are

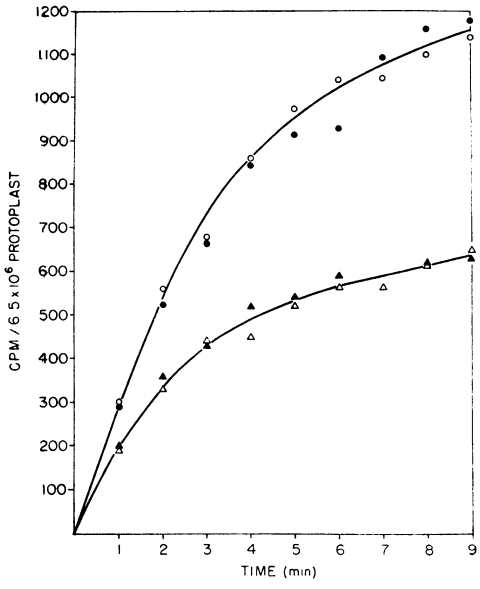

FIG. 3.—Effect of rifampin on RNA synthesis in isolated protoplasts. O, Total reaction mixture as described in *Methods;* ●, protoplasts preincubated with 10 μg/ml of rifampin; ▲, protoplasts preincubated with 250 μg/ml of rifampin; △, protoplasts preincubated with 500 μg/ml of rifampin.

labeled for four hours with adenine-8-^{14}C (0.01 μc/ml, 0.0002 μ*M*/ml) in the presence of rifampin, only the 25*S* and 18*S* peaks are labeled. As a result of the inhibition of rRNA synthesis, cells grown in the presence of rifampin lack chloroplast ribosomes as shown by electron microscopy (U. Goodenough, unpublished observation). These effects are not mimicked by growing cells in the presence of spectinomycin, an inhibitor of protein synthesis in the chloroplast (Surzycki, unpublished observations), and thus they are thought to be a direct consequence of rifampin inhibition of RNA synthesis.

Rifampin has no noticeable effect on DNA synthesis in either the chloroplast or the nucleus, for the nuclear and chloroplast DNA content is unchanged after four generations of growth in the presence of the drug.

Cells are unable to grow in liquid minimal media in the presence of 250 μg/ml of rifampin; lower concentrations of the drug do not inhibit phototrophic growth. However, cells will grow heterotrophically on an acetate-supplemented medium in the presence of rifampin at 250 μg/ml. Preliminary experiments show that cells grown under these conditions lose their ability to fix CO_2 by photosynthesis after four divisions. Rifampin is not an inhibitor of photosynthesis *per se;* there is no immediate effect on CO_2 fixation when rifampin is added to a control culture. Therefore, the effect of rifampin is presumably mediated through its effect on the chloroplast RNA-synthesizing machinery.

Discussion.—RNA polymerase located in the chloroplasts of *C. reinhardi* has been found to be different from the corresponding enzyme in the nucleus, since only the chloroplast enzyme is susceptible to inhibition by rifampin. The inhibition occurs *in vitro* as well as *in vivo*.

The contribution of the chloroplast-located DNA-dependent RNA polymerase to the over-all RNA synthesis of *C. reinhardi* is about 44 per cent in assays with intact cells; the same value is obtained in assays with purified protoplasts.

Since these estimations are based on the rate of adenine incorporation into RNA, they reflect the differences in the adenine content of both RNA's. If one assumes that the average adenine content of the RNA synthesized in the nucleus reflects the adenine content of nuclear DNA (35.7% AT)[15] and, similarly, that RNA synthesized in the chloroplast has an average adenine content equal to that of chloroplast DNA (67.3% AT),[15] one can calculate that the 44 per cent of RNA synthesis attributed to the chloroplast corresponds to 27 per cent of the total RNA synthesized by the cells. This number is somewhat higher than expected on the basis of the DNA content of the chloroplast, which constitutes only 15 per cent of the total DNA in the cell.[12] However, it is unlikely that at any given moment the entire nuclear genome is being transcribed, and indeed the above calculations indicate that only about 50 per cent of the nuclear DNA was open to transcription by RNA polymerase.

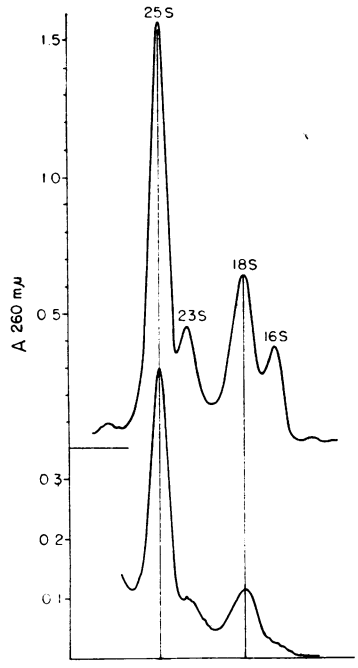

FIG. 4.—Polyacrylamide gel fractionation of nucleic acids isolated from cells grown for five generations heterotrophically in the absence (*top*) and the presence (*bottom*) of rifampin.

It seems probable that the similarity between chloroplast and bacterial DNA-dependent RNA polymerase, which has also been noticed by others,[16, 17] stems from similarities between the nature and state of the DNA templates on which these enzymes are working. The organization of the DNA in the chloroplast clearly resembles that in bacteria rather than that found in the nucleus. It therefore seems probable that chloroplast DNA-dependent RNA polymerase transcribes chloroplast DNA *in vivo*, not only by virtue of its localization in the organelle but also because it possesses a template specificity.

The inhibition of chloroplast RNA polymerase would result in the disappearance of all species of RNA transcribed from chloroplast DNA. Experiments carried out with *C. reinhardi* suggest that: (1) All functional cistrons for the chloroplast ribosomal RNA are located in chloroplast DNA.

(2) DNA polymerase, which is responsible for the replication of chloroplast DNA, is not synthesized on the chloroplast ribosomes, and the cistron for this enzyme is located in the nucleus. These conclusions are based on the finding that cells grown on rifampin contain a normal amount of chloroplast DNA per cell and lack chloroplast ribosomes.

(3) The information contained in chloroplast DNA, as well as all proteins that are synthesized on chloroplast ribosomes, is not vital for the growth and division of the cells, since the cells grow in the presence of rifampin when they are supplied with a carbon source. The information contained in the chloroplast DNA appears to concern only certain chloroplast functions and by no means assures

the continuity of the chloroplast as an organelle. Cells that were grown on the antibiotic for several generations do contain chloroplasts, although they show a large amount of internal disorganization (U. Goodenough, unpublished observations). Rifampin-grown cells are unable to fix CO_2 by photosynthesis.

The lack of ribosomes in cells grown on rifampin make it difficult to estimate which proteins are coded for by chloroplast DNA, for all proteins that require chloroplast ribosomes for their synthesis would be missing. There is no reason to assume that these ribosomes translate only messengers from chloroplast DNA or that the messenger originating in the chloroplast cannot be translated on cytoplasmic ribosomes.

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